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# Synthesis and Antitumor Activity of New Benzoheterocyclic Derivatives of Distamycin A

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The design, synthesis, and in vivo and in vitro antileukemic activity of a novel series of compounds (**13–22** and **34**), in which different benzoheterocyclic rings, bearing a nitrogen mustard or a benzoyl nitrogen mustard or an α-bromoacryloyl group as alkylating moieties, are tethered to a distamycin frame, are reported, and structure–activity relationships are discussed. The new derivatives were prepared by coupling nitrogen mustard-substituted, benzoyl nitrogen mustard-substituted, or α-bromoacryloyl-substituted benzoheterocyclic carboxylic acids **23–32** with desformyldistamycin (**33**) or in one case with its two-pyrrole analogue **35**. With very few exceptions, the activities of compounds bearing the same alkylating moiety are slightly affected by the kind of the heteroatom present on the benzoheterocyclic ring. All novel compounds, with one exception, showed in vitro activity against L1210 murine leukemia cell line comparable to or better than that of tallimustine. The compounds in which the nitrogen mustard and the α-bromoacryloyl moieties are directly linked to benzoheterocyclic ring showed potent cytotoxic activities ($IC_{50}$ ranging from 2 to 14 nM), while benzoyl nitrogen mustard derivatives of benzoheterocycles showed reduced cytotoxic activities, and one compound (**16**) of this cluster was the sole derivative devoid of significant activity. Compound **18**, a 5-nitrogen mustard *N*-methylindole derivative of distamycin, showed the best antileukemic activity in vivo, with a very long survival time (%T/C = 457), significantly increased in comparison to tallimustine (%T/C = 133), and was selected for further extensive evaluation. Arrested polymerase chain reaction and direct DNA fragmentation assays were performed for compound **18** and the structurally related compounds **13–17** and **19**. The results obtained have shown that both alkylating groups and oligopeptide frames play a crucial role in the sequence selectivity of these compounds.

## Introduction

There is currently interest in the study and development of low-molecular-weight sequence-selective agents interacting with double-stranded DNA. These molecules are oftenly based on natural products and have been investigated for their ability to interact selectively with the minor groove of DNA. One of the most studied minor groove binders is distamycin A.

Distamycin A (**1**) is a naturally occurring antibiotic,[1] characterized by the presence of an oligopeptidic pyrrolecarbamoyl frame ending with an amidino moiety, which reversibly binds to the minor groove of DNA with a strong preference for adenine-thymine (AT)-rich sequences containing at least four AT base pairs.[2] Distamycin A has been used as a DNA sequence-selective carrier of alkylating functions, leading to compounds which are substantially more cytotoxic than distamycin itself.

Mongelli and co-workers synthesized several semisynthetic analogues of distamycin A wherein the formyl group was replaced with different alkylating functions, such as benzoyl nitrogen mustard, nitrogen mustard, or α-bromoacryloyl moieties, obtaining the corresponding derivatives **2**[3] (tallimustine or FCE 24517), **3**,[4] and **4**,[3] respectively. Tallimustine (**2**) was selected as an antineoplastic drug candidate in view of its high activity against a wide spectrum of experimental tumors. However, since its clinical evaluation showed severe myelotoxicity, its development was discontinued.[5]

It is important to underline that, as occurs in the case of distamycin A and its four-pyrrole homologue **5**, the increase in the number of pyrrole units of the oligopeptidic frame in **2–4** led to derivatives **6–8**, respectively, which showed increased in vitro cytoxicity and in vivo antitumor activity.[3,4]

Recently, we have described[6] the synthesis and the activity of two isosteric derivatives, **9** and **10**, of **6** and **8**, respectively, in which the *N*-methylpyrrole directly linked to the alkylating moiety was replaced by an *N*-methylpyrazole. Both these isosteres **9** and **10** showed antitumor activity against L1210 leukemia, which was almost equivalent to that exhibited by **6** and **8**, respectively. It should be underlined that, on the contrary, the analogue **11** of compounds **6** and **9**, in which a *N*-methylimidazole replaced the pyrrole or pyrazole unit, respectively, appeared about 2 orders of magnitude less

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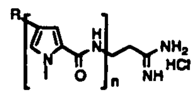
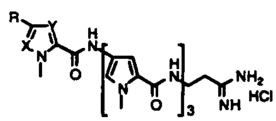


| Compound | n | R |
|---|---|---|
| **1** | 3 | NHCHO (Distamycin A) |
| **2** | 3 | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ |
| **3** | 3 | $(ClCH_2CH_2)_2N$ |
| **4** | 3 | $CH_2{=}CBrCONH$ |
| **5** | 4 | NHCHO |
| **6** | 4 | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ |
| **7** | 4 | $(ClCH_2CH_2)_2N$ |
| **8** | 4 | $CH_2{=}CBrCONH$ |

cytotoxic than its pyrrole or pyrazole counterparts. Moreover, the analogue **12** of compounds **8** and **10**, in which a *N*-methylimidazole replaced the pyrrole or pyrazole unit, respectively, showed in vitro cytoxicity against L1210 cells which was found to be equivalent to or slightly lower than that of **8** and **10**, the antileukemic activity in vivo being higher than that exhibited by **10**.



| Compound | X | Y | R |
|---|---|---|---|
| **9** | N | CH | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ |
| **10** | N | CH | $CH_2{=}CBrCONH$ |
| **11** | CH | N | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ |
| **12** | CH | N | $CH_2{=}CBrCONH$ |

Following these results, we report in this paper the synthesis and the biological evaluation of the new distamycin A derivatives **13–22**, in which the pyrrole ring bearing a nitrogen mustard, benzoyl nitrogen mustard, or α-bromoacryloyl alkylating moiety of compounds **6–8** has been replaced by different benzoheterocycles such as indole, *N*-methylindole, benzoimidazole, and benzofuran.[7a] Since pyrrole is susceptible to oxidative breakdown, these new derivatives have been prepared as potentially more stable minor groove binders aimed to improved the relative instability of the polypyrrolic skeleton. The choice of these benzoheterocycles can be also justified due to their importance in increasing the binding to DNA and the selectivity of alkylation of CC-1065 analogues such as U-71,184, adozelesin, and bizelesin.[7b]

## Chemistry

The synthetic route followed for the synthesis of derivatives **13–22** is outlined in Scheme 1. The key step was the coupling between benzoheterocyclic carboxylic acids **23–32** bearing the alkylating moieties and *N*-deformyldistamycin (**33**), obtained from distamycin A according to a reported procedure.[1a] For the preparation of compound **34**, the acid **31** was coupled with the known amino amidine **35**.[8]

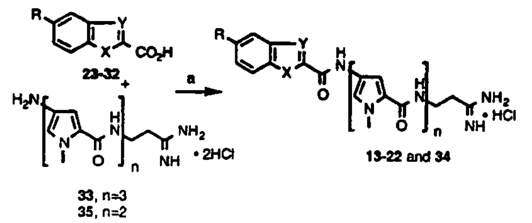
The condensations of the acylating agents **23–32** with deformyldistamycin (**33**) and of the acid **31** with **35** were performed using a slight excess (1.5 equiv) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) as coupling agent, in dry DMF as solvent, in the presence of Hunig's base, at room temperature and with identical reaction times (18 h). Compounds **13–22** and **34** were prepared in acceptable yields, after purification by silica gel flash chromatography.

The synthesis of heterocyclic acids **23–26**, bearing the benzoic acid mustard (BAM), was performed by coupling ethyl 5-aminoindole-2-carboxylate (**36**),[9] ethyl 1-methyl-4-aminoindole-2-carboxylate (**37**),[10] ethyl 5-aminobenzofuran-2-carboxylate (**38**), and ethyl 5-amino-1(3*H*)-benzoimidazole-2-carboxylate (**39**), respectively, to *p*-[bis(2-chloroethyl)amino]benzoyl chloride (**40**)[11] to give the corresponding amido esters **41–44**, which after purification by flash chromatography were submitted to mild alkaline hydrolysis to yield the corresponding acids **23–26**, respectively (Scheme 2).

For the synthesis of acid intermediates **27–29**, the amino esters **36–38** were converted in good yields to the corresponding *N,N*-bis(2-hydroxyethyl) derivatives **45–47**, respectively, by reaction with a large excess (5 equiv) of ethylene oxide in methanol. Subsequent treatment with phosphorus oxytrichloride ($POCl_3$) afforded the corresponding dichloro nitrogen mustards **48–50**, which were transformed into the desired acids **27–29** by acid hydrolysis (Scheme 3).

The synthetic approach employed for the preparation of the α-bromoacryloyl derivatives **30–32** shown in Scheme 4 was carried out by conversion of the nitro carboxylic acids **51**,[12] **52**,[10] and **53**[13] into the corresponding *tert*-butyl esters **54–56** in good yield by treating **52**

**Scheme 1**[a]

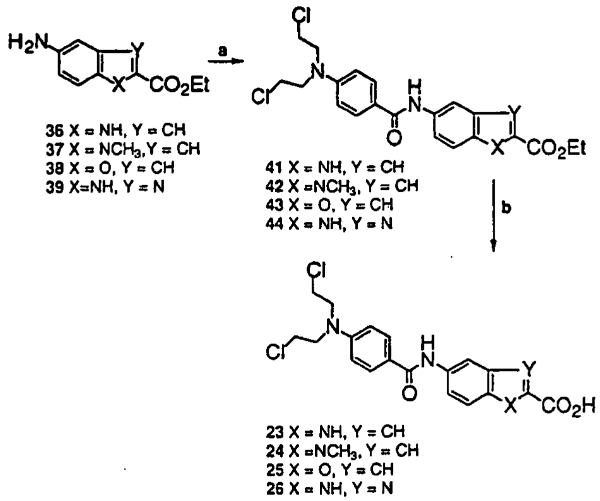


[a] Reagents: (a) EDC, Hunig's base, DMF, 18h, r.t.

| n | R | X | Y | Product |
|---|---|---|---|---|
| 3 | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ | NH | CH | **13** |
| 3 | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ | $NCH_3$ | CH | **14** |
| 3 | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ | O | CH | **15** |
| 3 | $4\text{-}[(ClCH_2CH_2)_2N]C_6H_4CONH$ | NH | N | **16** |
| 3 | $(ClCH_2CH_2)_2N$ | NH | CH | **17** |
| 3 | $(ClCH_2CH_2)_2N$ | $NCH_3$ | CH | **18** |
| 3 | $(ClCH_2CH_2)_2N$ | O | CH | **19** |
| 3 | $CH_2{=}CBrCONH$ | NH | CH | **20** |
| 3 | $CH_2{=}CBrCONH$ | $NCH_3$ | CH | **21** |
| 3 | $CH_2{=}CBrCONH$ | O | CH | **22** |
| 2 | $CH_2{=}CBrCONH$ | $NCH_3$ | CH | **34** |

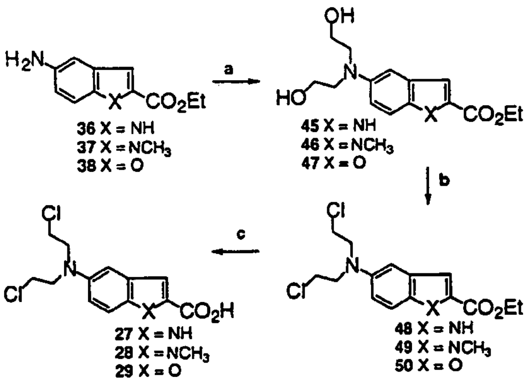
[a] Reagents: (a) EDC, Hunig's base, DMF, 18 h, rt.

**Scheme 2**[a]



[a] Reagents: (a) **40**, $Et_3N$, $CH_2Cl_2$, 18 h, rt; (b) KOH, water/dioxane, 3 h, rt.

**Scheme 3**[a]

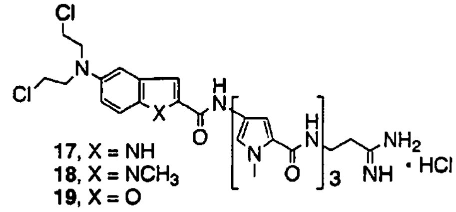


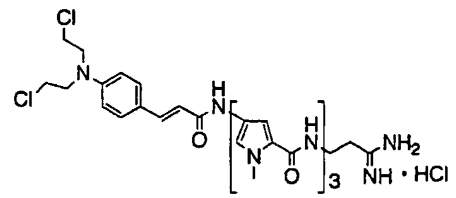
[a] Reagents: (a) ethylene oxide; (b) $POCl_3$, toluene, rt, 1 h; (c) 37% HCl in water, rt, 3 h.

and **53** with carbonyldiimidazole (CDI) and then with *tert*-butyl alcohol[14] and **51** with *tert*-butyl bromide[15] (Scheme 4). The amino compounds **57–59**, obtained by catalytic hydrogenation (10% Pd–C) of the corresponding nitro *tert*-butyl esters **54–56**, after treatment with the α-bromoacrylic acid in the presence of 2 equiv of EDC afforded **60–62**, respectively. Subsequent treatment with trifluoroacetic acid (TFA) at room temperature afforded the carboxylic acids **30–32**.

## Results and Discussion

**In Vitro and in Vivo Antitumor Activity.** The in vitro cytotoxic activity of the synthesized compounds **13–22** and **34** was evaluated against the L1210 murine leukemia cell line and in L1210 sublines resistant to tallimustine (L1210/tallimustine) and to doxorubicin (L1210/DX). The cytotoxic activity of the synthesized compounds has been compared with that of distamycin A (**1**), tallimustine (**2**), and the distamycin derivatives **3–12** and **64**. This latter compound is a previously reported cynnamoyl nitrogen mustard derivative[16] of distamycin A, a vinylogue of tallimustine. Compounds **17–19** should be considered as "conformationally constrained" forms of the cinnamic mustard derivative **64** of distamycin A, where the benzoheterocyclic unit incorporating both the phenyl and the vinylic double bond of the cinnamic moiety confers rigidity to the alkylating region of the molecule.





**64**

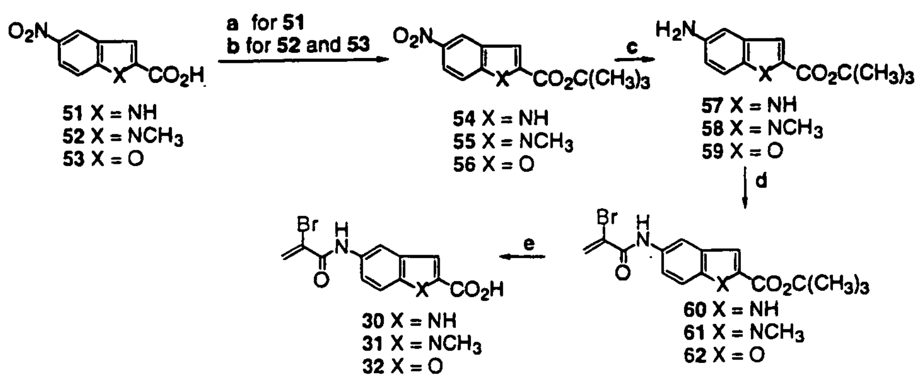
The compounds **13–22** and **34** were also tested in vivo against L1210 murine leukemia cells, and some representative molecules (**18** and **21**) were also tested on M5076 murine reticulosarcoma. The results obtained are summarized in Table 1.

Derivatives **13–15** containing the BAM as alkylating moiety showed significant cytotoxicity against L1210 cell line with $IC_{50}$ values comparable to that reported for tallimustine (**2**) but lower than those reported for both its pyrrole **6**[3] and pyrazole **9**[6] homologues. Derivatives **13–15** were at least 30-fold more cytotoxic than the benzoimidazole derivative **16**, which showed antiproliferative activity similar to that reported for the imidazole distamycin analogue **11**.[6] The poor activity of **16** may be related to a different DNA recognition sequence, due to the possible role of the N(3) lone pair of the benzoimidazole ring[17] which may be capable to accommodate hydrogen bonding with the guanine 2-amino group and thus provide for GC selectivity. This could alter the strict preference for AT sequence allowing GC recognition and then mixed AT/GC sequences.

Compounds **13–15** not only exhibited good activity in in vitro experiments but also showed a significant increase in survival time (%T/C) of mice bearing the lymphocytic leukemia model (L1210). Derivatives **14** and **15** were 8- and 2-fold less potent than tallimustine (optimal nontoxic dose (OD): OD = 25 mg/kg for **14**, 6.25 mg/kg for **15** vs OD = 3.13 mg/kg for tallimustine), while **13** showed a potency similar to that of tallimustine, with a median survival time slightly higher (%T/C = 163 for **13** vs %T/C = 133 for tallimustine). In this series of BAM tetrapeptides **13–15**, the compound **14**, which was the less potent in vivo, possessed a %T/C value comparable to that of tallimustine (%T/C = 183 for **13** vs %T/C = 133), while **15** showed a lower value (%T/C = 150). Moreover, compounds **13–15** were 2–4-fold less cytotoxic than the pyrrole **6** and pyrazole **9** counterparts.

The nature of the alkylating group had a great effect on the cytoxicity of compounds having the same oligopeptide skeleton. In fact, the presence of a nitrogen mustard moiety directly linked to the benzoheterocyclic ring, as in the case of compounds **17–19**, instead of a nitrogen benzoyl mustard moiety, as in the case of

**Scheme 4**[a]




[a] Reagents: (a) $(CH_3)_3C{-}Br$, $K_2CO_3$, BTEAC, DMF; (b) CDI, *tert*-butyl alcohol, DBU, DMF; (c) $H_2$, Pd–C; (d) α-bromoacrylic acid, EDCI, DMF; (e) $CF_3CO_2H$, rt, 1 h.

**Table 1.** In Vivo and in Vitro Activity of Distamycin Derivatives against L1210 Murine Leukemia[a]

| | in vitro L1210 | in vivo L1210 | | | |
|---|---|---|---|---|---|
| | | ip | | iv | |
| compd | $IC_{50}$ (nM ± SE) | OD (mg/kg) | %T/C | OD (mg/kg) | %T/C |
| distamycin A[3] | 10069 ± 1647 | 200 | 113 | nd | nd |
| tallimustine (2)[3] | 68.5 ± 6.6 | 3.1 | 175 | 3.1 | 133 |
| **3**[4] | 3.9 ± 0.6 | 1.6 | 138 | 0.78 | 117 |
| **4**[3] | 79.6 ± 14 | 12.5 | 175 | nd | nd |
| **5**[3] | 445 ± 75 | nd | nd | nd | nd |
| **6**[3] | 19.0 ± 0.6 | 0.4 | 138 | nd | nd |
| **7**[4] | 0.081 ± 0.01 | 0.39 | 188 | 0.39 | 133 |
| **8**[3] | 6.3 ± 1.34 | 3.1 | 725 | 1.6 | 200 |
| **9**[6] | 34.5 ± 4.6 | 3.1 | 144 | nd | nd |
| **10**[6] | 13.3 ± 0.54 | 6.2 | 200 | nd | nd |
| **11**[6] | 2372 ± 157 | 6.2 | 125 | nd | nd |
| **12**[6] | 46.9 ± 13 | 6.2 | 163 | nd | nd |
| **13** | 95.3 ± 28 | nd | nd | 3.1 | 163 |
| **14** | 61.7 ± 2.49 | nd | nd | 25 | 183 |
| **15** | 43.5 ± 7 | nd | nd | 6.2 | 150 |
| **16** | 1422 ± 235 | nd | nd | nd | nd |
| **17** | 8.0 ± 2.6 | nd | nd | 0.39 | 174 |
| **18** | 14.7 ± 2.4 | nd | nd | 1.6 | 457 |
| **19** | 3.7 ± 1.5 | nd | nd | 0.39 | 200 |
| **20** | 4.1 ± 1.3 | nd | nd | 12.5 | 213 |
| **21** | 2.4 ± 0.39 | nd | nd | 6.2 | 192 |
| **22** | 6.1 ± 0.93 | nd | nd | 0.78 | 117 |
| **34** | 15.3 ± 4.31 | nd | nd | 3.1 | 133 |
| **64**[16] | 9.5 ± 2.71 | nd | nd | 6.2 | 267 |

[a] $IC_{50}$ = 50% inhibitory concentration as the mean ± SE from dose–response curves of at least three experiments; ip = treatment was performed intraperitoneally on day 1 after tumor ip transplant; iv = treatment was performed intravenously on day 1 after tumor iv transplant; OD = optimal dose, optimal nontoxic dose < $LD_{10}$ (weight loss < 20% with respect to the starting weight); %T/C = median survival time of treated vs untreated mice × 100; nd = not determined.

compounds **13–16**, proved to be effective in increasing the cytotoxic activity by a factor of up to 10.

Especially noteworthy was the compound **18**, which although 2- or 4-fold less active in vitro ($IC_{50}$ = 14.7 nM for **18** vs $IC_{50}$ = 8.04 nM for **17**, 3.75 nM for **19**) and 4-fold less potent in vivo with respect to **17** and **19** (OD = 1.56 mg/kg for **18** vs OD = 0.39 mg/kg both for **17** and **19**), exhibited a %T/C of 457, which was almost 3 times higher than that of **17** (%T/C = 174) and **19** (%T/C = 200). Compounds **17–19**, although 2 orders of magnitude less cytotoxic than the nitrogen mustard derivative **7** with four pyrrole units, showed superior activity in vivo (%T/C = 174, 457, and 200 for **17–19**, respectively, vs %T/C = 133 for **7**).

As reported for other series of distamycin derivatives,[18] compounds **20–22**, bearing the α-bromoacryloyl moiety, gave better results in terms of activity both in vitro and in vivo, in comparison to the corresponding derivatives **13–15**, which possess the BAM function as the alkylating moiety. In fact compounds **20–22** were at least 10-fold more cytotoxic than **13–15** and, with the exception of **22**, showed %T/C values which were higher than those reported for the benzoyl mustard counterparts.

In the same series **20–22**, although the indole derivative **20** showed the same cytotoxicity as the benzofuran counterpart **22**, the latter compound was 15-fold less potent in vivo and produced an increased survival time 2-fold higher than that for **20** (OD = 0.78 mg/kg, %T/C = 117 for **22** vs OD = 12.5 mg/kg, %T/C = 213 for **20**). From the comparison of the compounds bearing the nitrogen mustard or α-bromoacryloyl unit as the alkylating moiety, it was found that derivatives **20** and **21** were more cytotoxic, but less potent in vivo, than the *N*-nitrogen mustard counterpart **17** and **18**, since **22** was both less cytotoxic and potent than **19**. The same compounds **20–22** maintained cytotoxicity substantially equivalent to that of the pyrrole **8** and pyrazole **10** analogues, but much higher with respect to the imidazole derivative **12**.

A fairly marked correlation between antitumor activity and length of the polypyrrolic skeleton was observed for compounds **21** and **34**, where the derivative **34** with two *N*-methylpyrrolic units was 5-fold less cytotoxic than the corresponding pyrrole homologue **21**. This is in agreement with the hypothesis that DNA binding, which depends on the multiplicity of interactions between the pyrrolecarbamoyl units and AT-rich sequences of DNA, is crucial for cytotoxicity.[19] Derivative **34**, despite a potency 2-fold higher than that of **21** (OD = 6.25 vs 3.13 mg/kg), was slightly less effective than the latter in vivo (T/C% = 192 vs 133 for **21** and **34**, respectively).

In a preliminary in vivo evaluation against the M5076 solid tumor, compound **21** was almost 4-fold less toxic than **18** (OD = 0.93 mg/kg for **18** vs OD = 3.13 mg/kg for **21**), with an increased %T/C slightly lower (%T/C = 124 for **18** vs %T/C = 154 for **21**), while both compounds showed the same trend of inhibition of tumor growth (%TI = 92 for **18** vs %TI = 96 for **21**).

In Table 2 the resistance index (RI) values of **1–22**, **34**, and **64** on leukemia cell lines resistant to doxoru-

**Table 2.** Cytotoxicity and Resistance Index (RI) of Distamycin Derivatives against L1210 Leukemia Cells Resistant to Doxorubicin (DX) and Tallimustine[a]

| | L1210/DX | | L1210/tallimustine | |
|---|---|---|---|---|
| compd | $IC_{50}$ ($\mu$M ± SE) | RI | $IC_{50}$ ($\mu$M ± SE) | RI |
| distamycin A[3] | 459 ± 50.1 | 45.6 | 142 ± 13.4 | 14.1 |
| tallimustine (2)[3] | 2.6 ± 0.2 | 38.5 | 0.55 ± 0.06 | 8 |
| 3[4] | nd | nd | nd | nd |
| 4[3] | 5.4 ± 1 | 68.0 | 12.4 ± 2.5 | 156 |
| 5[3] | 78.1 ± 6.3 | 175.4 | 75.6 ± 42.3 | 169.9 |
| 6[3] | nd | nd | 1.24 ± 0.3 | 65.4 |
| 7[4] | nd | nd | nd | nd |
| 8[3] | 0.024 ± 0.0002 | 3.8 | 0.17 ± 0.013 | 27.0 |
| **9** | 11.3 ± 1.2 | 326.2 | 10.4 ± 1.2 | 302.3 |
| **10** | 0.093 ± 0.017 | 7 | 0.55 ± 0.13 | 41.4 |
| **11** | 4.7 ± 1.2 | 2 | 5 ± 0.082 | 2.1 |
| **12** | 0.73 ± 0.069 | 15.5 | 0.65 ± 0.14 | 13.9 |
| **13** | 0.54 ± 0.039 | 5.7 | 0.88 ± 0.039 | 9.2 |
| **14** | 0.42 ± 0.041 | 6.8 | 1.2 ± 0.13 | 19 |
| **15** | 0.34 ± 0.01 | 7.8 | 12.9 ± 0.58 | 295.8 |
| **16** | 3.8 ± 0.59 | 2.7 | nd | nd |
| **17** | 0.059 ± 0.0007 | 7.3 | 0.093 ± 0.00021 | 11.6 |
| **18** | 0.11 ± 0.01 | 7.4 | 0.078 ± 0.002 | 5.3 |
| **19** | 0.07 ± 0.01 | 18.7 | 0.085 ± 0.008 | 22.8 |
| **20** | 0.036 ± 0.0056 | 8.7 | 0.14 ± 0.03 | 34.9 |
| **21** | 0.043 ± 0.015 | 18 | 0.15 ± 0.036 | 64.8 |
| **22** | 0.039 ± 0.002 | 6.4 | 0.21 ± 0.019 | 33.8 |
| **34** | 0.041 ± 0.0009 | 2.7 | 0.15 ± 0.076 | 10.1 |
| **64**[16] | 0.13 ± 0.10 | 17.6 | 0.11 ± 0.023 | 8.00 |

[a] $IC_{50}$ = 50% inhibitory concentration as the mean ± SE from dose–response curves of at least three experiments; RI (resistance index) = ratio between $IC_{50}$ values on resistant cells and sensitive cells.

bicin (Dx) and tallimustine are reported. The results obtained show that, for the newly synthesized compounds **13–22** and **34**, only **13**, **17**, **18**, and **34** display low RI values for leukemia cell lines resistant to doxorubicin and tallimustine. For each new derivative, with the exception of **18**, which was more active in the tallimustine-resistant L1210 leukemia line, it was interesting to note that the RI values in L1210/tallimustine cells were at least 2-fold higher than that reported in L1210/DX cells, showing that neither the alkylating moiety nor the benzoheterocycle were involved in the resistance mechanism.

It is also interesting to note that in the comparison between compounds **13** and **14**, **17** and **18**, **20** and **21** which bear the same alkylating moieties, N-methylation of the indole nucleus had an important effect only for the %T/C value of **18**. Finally, nitrogen mustard and α-bromoacryloyl derivatives showed comparable cytotoxicity against L1210 murine leukemia cells, superior to that reported for BAM derivatives with the same oligopeptidic frame.

**Arrested Polymerase Chain Reaction (PCR) and Direct DNA Fragmentation Assay.** To determine the influence of the alkylating group and the oligopeptide skeletons on sequence selectivity, two series of derivatives, **13–16** and **17–19**, were compared, employing molecular studies, such as arrested PCR and a direct DNA fragmentation assay.

First, we determined the effect of compounds **13–16** and **17–19** on PCR-mediated amplification of two genomic sequences: one rich in A+T, the other rich in G+C. The human estrogen receptor (ER) gene (A+T/G+C = 3.46) and the human Ha-ras oncogene (A+T/G+C = 0.37) were chosen as model systems, as they are suitable for use in determining sequence-selective bind-

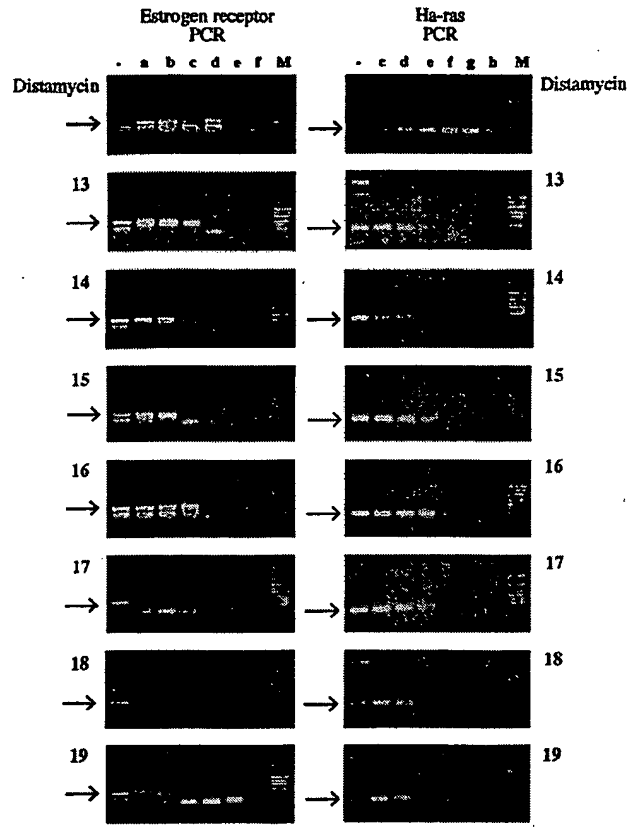



**Figure 1.** Effects of distamycin and distamycin analogues **13–19** on PCR-mediated amplification of estrogen receptor (ER; left side of the panel) and Ha-ras (right side of the panel) genomic sequences. For PCR-mediated amplification the target DNA was 20 ng of human genomic DNA or 2 ng of pBLCAT1-ERCAT8; PCR buffer, Taq DNA polymerase, and the four dNTPs were added as elsewhere described.[21] Before PCR, target DNA was incubated in the absence (−) or presence (a–h) of DNA-binding compounds (a = 1 $\mu$M, b = 2 $\mu$M, c = 5 $\mu$M, d = 10 $\mu$M, e = 20 $\mu$M, f = 50 $\mu$M, g = 100 $\mu$M, h = 200 $\mu$M) for 10 min. Conditions of Ha-ras PCR were: denaturation, 92 °C, 45 s; annealing, 62 °C, 45 s; elongation, 72 °C, 30 s (25 cycles). Conditions of ER PCR were: denaturation, 92 °C, 45 s; annealing, 55 °C, 1 min; elongation, 72 °C, 1 min (25 cycles). Amplified DNA was analyzed by electrophoresis on 2.5% agarose, 0.5 mg/mL ethidium bromide. Specific ER and Ha-ras PCR products are arrowed. Specificity of ER and Ha-ras PCR products was confirmed by Southern blotting and hybridization to specific $^{32}$P-labeled probes as well as isolation of the arrowed PCR products and direct sequencing (ref 22 and data not shown).

ing of DNA-binding compounds when amplified by PCR.[20,21] We have recently published the nucleotide sequence of a 3.2-kb genomic region located upstream of the estrogen receptor sequence[22] originally designated exon-1 and demonstrated that this region contains A+T-rich sequences recognized by distamycin A and distamycin analogues.[23] The human Ha-ras oncogene sequence, on the other hand, is G+C-rich and therefore interacts with low efficiency with distamycin, being, on the contrary, efficiently recognized by G+C-selective binders, such as chromomycin and mithramycin.[20] Figure 1 shows a representative example of the PCR experiment performed using distamycin A (**1**) and distamycin analogues; Table 3 shows all the results obtained, expressed as the inhibitory activity ($IC_{50}$) of

**Table 3.** Inhibitory Activity ($IC_{50}$) of Distamycin Derivatives on the Generation of ER and Ha-ras PCR Products

| | PCR $IC_{50}$[a] ($\mu$M) | | | PCR $IC_{50}$[a] ($\mu$M) | |
|---|---|---|---|---|---|
| compd | ER | Ha-ras | compd | ER | Ha-ras |
| distamycin A | 20 | 200 | **16** | 10 | 50 |
| **13** | 7.5 | 15 | **17** | 1 | 20 |
| **14** | 5 | 15 | **18** | 1 | 20 |
| **15** | 3 | 20 | **19** | 3 | 15 |

[a] Inhibitory concentration (mM) necessary to obtain 50% inhibition of generation of PCR products of human Ha-ras and ER sequences.

distamycin derivatives and the generation of ER and Ha-ras PCR products.

In agreement with already published reports,[20,21] distamycin A (**1**) inhibits ER PCR (Figure 1, left side of the panel) but exhibits lower capacity to inhibit Ha-ras PCR (Figure 1, right side of the panel), thus confirming a selective binding of distamycin to A+T-rich gene sequences. $IC_{50}$ of distamycin was found to be 20 $\mu$M in the case of ER PCR and over 200 $\mu$M in the case of Ha-ras PCR. By contrast, compounds **13**–**15** and **17**–**19** are effective inhibitors of both ER and Ha-ras PCR (Figure 1), maintaining however sequence selectivity for ER A+T-rich gene sequences (see Table 3 for semiquantitative analysis). These data suggest that sequence-selective binding typical of distamycin is to some extent maintained by these compounds. As reported in Table 3, when compounds **13**–**15** are compared to the corresponding **17**–**19**, no major differences were found with respect to inhibitory activity on the generation of Ha-ras PCR products. By contrast, compounds **17**–**19** were found to be consistently more active than the corresponding **13**–**15** in inhibiting the generation of ER PCR products (Figure 1 and Table 3).

To further investigate sequence selectivity of compounds **13**–**16** and **17**–**19**, arrested PCR fragments generated using a $^{32}$P-labeled ER PCR primer were analyzed by gel electrophoresis and the sites of arrest identified. The results obtained are shown in Figure 2 and demonstrate that the sequence selectivity of compounds **13**–**16** and **17**–**19** is readily observed. The nucleotide sequences of the sites of arrest are also shown in Table 4.

Compounds **15** and **19** were the most active compounds (inhibition of generation of full-length PCR product was obtained at 2 $\mu$M concentration). The analysis of the nucleotide sequences corresponding to the sites of arrest of the PCR demonstrate that the arrested sites (5′-AGTTAAAA-3′ and 5′-TAAT-3′) were found when the arrested PCR was performed in the presence of all the compounds tested. The arrested site 5′-TTTAACTT-3′ was preferentially found in the presence of compounds **14** and **15**, also being clearly detectable in the presence of compounds **13** and **17**–**19**. The arrested site 5′-ATGTGTGTGTGTA-3′ was preferentially found in the presence of compounds **18** and **19**.

A first conclusion gathered from the data shown in Figure 2 is that both alkylating groups and oligopeptide skeletons play a crucial role in determining the sequence selectivity of these compounds. Direct DNA fragmentation assays (Figure 3) confirmed these conclusions. In particular, it should be noted that compounds **17**–**19** are more active in this assay than compounds **13**–**16**. In addition, comparative analysis of the lanes corresponding to compounds **17**–**19** shows that a different pattern of fragmentation is generated after the direct DNA fragmentation assay (see the fragments corresponding to the site 5′-TATGTACGTGTGC-3′), suggesting differences of binding activity of compounds **17**–**19**. Further experiments employing other genomic experimental systems exhibiting similar GT-rich nucleotide sequences will be necessary in order to determine whether this observation could be generalized. In any case, it should be emphasized that major arrested sites of PCRs (Figure 2) extensively overlap with the nucleotide sequences corresponding to the fragments generated when the direct DNA fragmentation assay was performed (Figure 3).

Furthermore, it should be noted that a good relationship exists between activity in the arrested PCR assay (Table 3) and in vitro cytotoxicity (Table 1). For instance, compounds **17**–**19** were found to be more active than compounds **13**–**15** in both the in vitro cytotoxicity tests and the arrested PCR assays. Accordingly, compound **19** was found to be more active than **17** and **18** in both the in vitro cytotoxicity tests and the arrested PCR and direct DNA fragmentation assays. Compound **16** was consistently found to be less active than compounds **13**–**15**.

## Conclusions

All the novel compounds, with the exception of compound **16**, showed in vitro activity against L1210 murine leukemia cell line comparable to or better than that of tallimustine. Compounds in which the nitrogen mustard and the α-bromoacryloyl moieties are directly linked to the benzoheterocyclic ring showed very high equipotent activities, ranging from 2 to 14 nM, while benzoyl nitrogen mustard derivatives of benzoheterocycles showed lower activities, with one compound (**16**) of this cluster being the only derivative devoid of significant activity. Despite the fact that in vivo antileukemic activity was found to be generally poorly correlated with cytotoxicity, the exceptional antileukemic activity of **18** is noteworthy. Therefore, compound **18** has been selected for further extensive evaluation on murine solid tumors and human xenograft. The data obtained in arrested PCR and direct DNA fragmentation assays suggest that both the alkylating groups and the oligopeptide frames play a crucial role in determining the sequence selectivity of these compounds. In conclusion, the results presented in this paper suggest that the synthesis of various derivatives of distamycin A may be a useful approach to cancer chemotherapy, leading to the production of structurally related compounds exhibiting significant antitumor activity.

## Experimental Section

**Chemical Materials and Methods. General Procedure.** All reactions were carried out under argon atmosphere, unless otherwise described. Standard syringe techniques were applied for transferring anhydrous solvents. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography on silica gel (precoated $F_{254}$ Merk plates), the spots were examined with UV light and visualized with aqueous $KMnO_4$. $^1$H NMR spectra were recorded in the given solvent with a Bruker AC 200 spectrometer. Chemical shifts are reported as $\delta$ values in ppm. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), br (broad), and m (multiplet). Melting

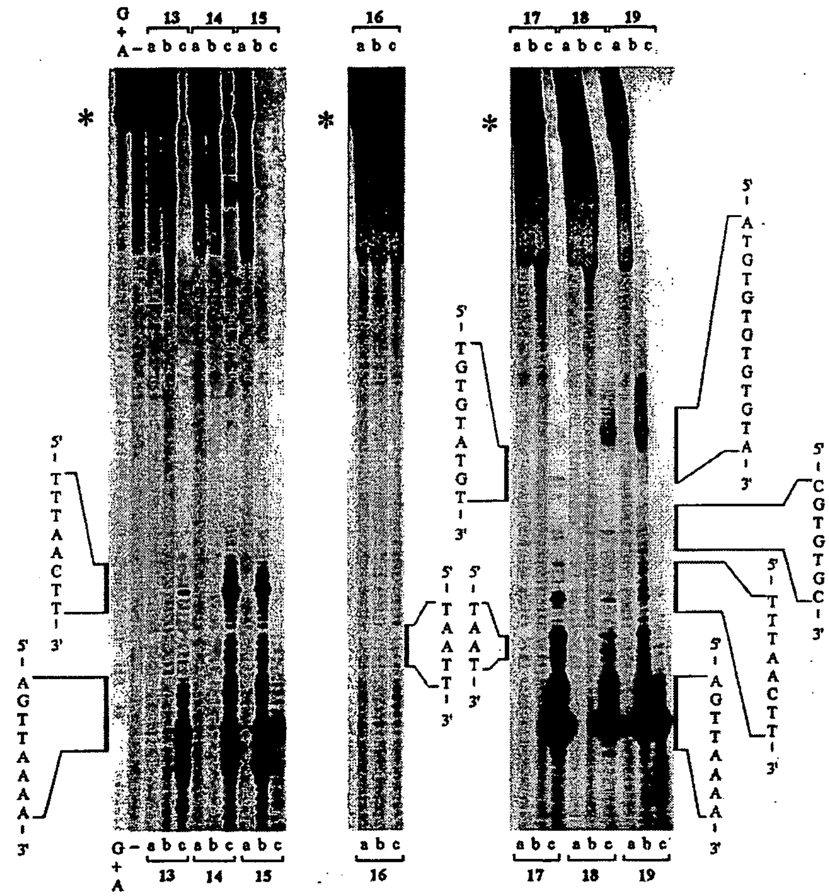


**Figure 2.** Arrested PCR: analysis of arrested sites. In this experiment, ER target DNA was prepared by PCR. After incubation in the absence (−) or presence of (a) 0.5, (b) 2, and (c) 10 μM DNA-binding compounds, a further 10 cycles of PCR were performed with a $^{32}P$-labeled ER reverse PCR primer. After PCR, the samples were heated at 92 °C for 5 min and layered onto a sequencing gel. The nucleotide sequences of the sites of PCR arrest are indicated. Asterisks indicate full-sized PCR products. At these compound concentrations distamycin A generates no appreciable sites of arrest of ER PCR (data not shown).

**Table 4.** Arrested PCR: Sequence Recognition by Distamycin Derivatives

| compd | nucleotide sequence |
|---|---|
| **18** and **19** | 5′-ATGTGTGTGTGTA-3′ |
| **17** | 5′-TGTGTATGT-3′ |
| **17**−**19** | 5′-CGTGTGC-3′ |
| **13**−**15** and **17**−**19** | 5′-TTTAACTT-3′ |
| **13**−**19** | 5′-TAATT-3′ |
| **13**−**19** | 5′-CAGTTAAAA-3′ |

points (mp) were determined using a Buchi-Tottoli apparatus and are uncorrected. All products reported showed $^1H$ NMR spectra in agreement with the assigned structures. Mass spectra were recorded on a Nermag R10,10C spectrometer. Elemental analyses, conducted by the Microanalytical Laboratory, Chemistry Department, University of Ferrara, were within 0.4% of the theorical values calculated for C, H, Br, Cl, and N. Column chromatography was carried out Merck silica gel (230−240 mash). All compounds obtained commercially were used without further purification. Organic solutions were dried over anhydrous $MgSO_4$. Methanol was distilled from magnesium turnings, dioxane was distilled from calcium hydride, and anhydrous DMF was distilled from calcium chloride and stored over molecular sieves (3 Å). In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used.

**General Procedure A for the Synthesis of 38 and 39.** A solution of the appropriate nitro ester (2 mmol) in 10 mL of a mixture of MeOH/dioxane (1:1, v/v) was hydrogenated over 50 mg of 10% Pd/C at 60 psi for 5 h. The catalyst was removed by filtration; the filtrate was concentrated to give a green oil which was precipitated from EtOAc/petroleum ether and used without purification for the next step.

**General Procedure B for the Synthesis of BAM Esters 41−44.** A solution of *p*-[*N*,*N*-bis(2-chloroethyl)amino]benzoyl chloride (**40**) (310 mg, 1.1 mmol) in anhydrous $CH_2Cl_2$ (5 mL) was added dropwise in small portions to a mixture of amino ester **36**−**39** (1 mmol) and triethylamine (0.14 mL, 1 mmol) in anhydrous $CH_2Cl_2$ (5 mL) cooled to 0 °C. The reaction mixture was stirred at room temperature for 18 h and concentrated under reduced pressure yielding a brown solid, which was dissolved in EtOAc (20 mL) and washed with 2 N hydrochloric acid (2 × 10 mL). The organic layer was dried ($Na_2SO_4$) and concentrated and the resulting residue precipitated from EtOAc/petroleum ether to give **41**−**44**.

**General Procedure C for the Synthesis of 23−26.** A well-stirred solution of **41**−**44** (1 mmol) in dioxane (4 mL) was treated with 2 N KOH in water (1 mL) and stirred at room temperature for 3 h. The clear solution was evaporated to remove dioxane, diluited with water (5 mL), cooled on an ice−water bath and acidified with 2 N hydrochloric acid to pH 2. The aqueous suspension was extracted with EtOAc (2 × 10 mL) and the organic layers were combined, dried ($Na_2SO_4$) and concentrated. The resulting residue was precipitated from EtOAc−hexane to give the product **23**−**26**.

**General Procedure D for the Synthesis of Intermediates 45−47.** To a solution of amino ester **36**−**38** (1 mmol) in MeOH (10 mL) cooled at −10 °C was added cold ethylene oxide (2.5 mL). The reaction flask was sealed and allowed to reach room temperature overnight. Methanol and excess of ethylene oxide were removed by evaporation and the crude residue purified by flash chromatography on silica gel.

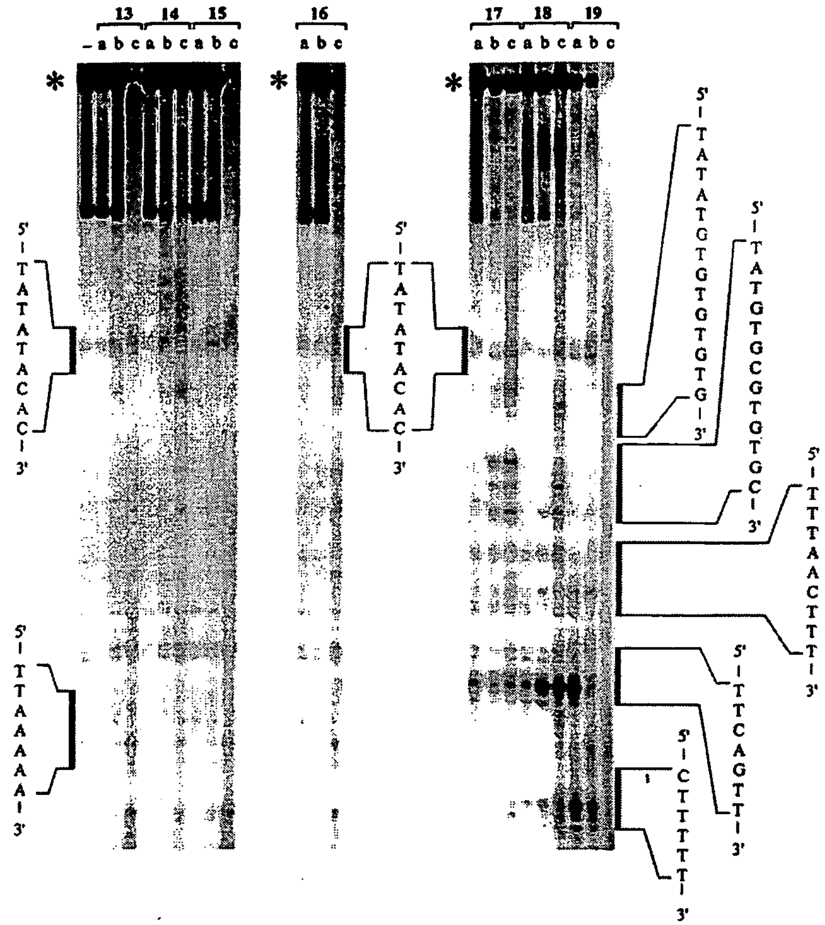



**Figure 3.** DNA cross-linking assay. In this experiment a $^{32}$P-labeled ER PCR product was produced using a $^{32}$P-labeled ER reverse PCR primer. After production of the ER PCR probe, an aliquot was incubated in 50 $\mu$L in the absence (−) or presence of (a) 0.5, (b) 2, and (c) 10 $\mu$M DNA-binding compounds. After 5 h incubation at room temperature the samples were heated at 90 °C for 30 min, ethanol-precipitated, and analyzed by electrophoresis on a sequencing gel. The nucleotide sequences corresponding to DNA fragmentation are indicated. Asterisks indicate full-sized PCR products.

**General Procedure E for the Synthesis of the Nitrogen Mustard Esters 48–50.** The compound **45–47** (1 mmol) was cooled in an ice bath and 2 mL of phosphorus oxychloride (20 mmol, 2 mL) was added dropwise. The solution was heated at 100 °C for 1 h, the solvent evaporated under vacum, and then the residue dissolved in EtOAc (8 mL) and washed with water (3 mL). The organic phase was dried ($Na_2SO_4$) and concentrated and the crude product purified by flash chromatography on silica gel.

**General Procedure F for the Synthesis of Nitrogen Mustard Carboxylic Acids 27–29.** The nitrogen mustard **48–50** (1 mmol) was dissolved in 36% hydrochloric acid (4 mL) and heated at 100 °C for 3 h. The solution was cooled at room temperature, diluted with water (10 mL) and extracted with EtOAc (2 × 20 mL). The recombined organic phases were dried ($Na_2SO_4$) and evaporated in vacuo and the residue was purified by flash chromatography on silica gel.

**General Procedure G for the Synthesis of Nitro *tert*-Butyl Esters 55 and 56.** A solution of nitro acid **52–53** (5 mmol) in dry DMF (10 mL) cooled at 0 °C was treated with CDI (1.1 g, 6.5 mmol). After 1 h at room temperature, *tert*-butyl alcohol (1 mL) and DBU (0.9 mL) were added. The mixture was stirred for 2 h and poured in cooled water (50 mL) and the resulting crystalline solid collected and washed with water, affording the desired nitro *tert*-butyl ester **55–56**.

**General Procedure H for the Reduction of Nitro *tert*-Butyl Esters 54 and 56.** The nitro *tert*-butyl ester **54–56** (5 mmol) was reduced over 10% Pd/C (100 mg) in a mixture of dioxane (10 mL) and methanol (10 mL) at room temperature under 55 psi pressure. After reduction, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue, after purification by flash chromatography, was dissolved in EtOAc and precipitated with an excess of petroleum ether.

**General Procedure I for the Synthesis of Bromoacrylamido *tert*-Butyl Esters 60–62.** To a solution of α-bromoacrylic acid (600 mg, 4 mmol) in dry DMF (5 mL) was added a solution of EDC (383 mg, 2 mmol) in dry DMF (5 mL) slowly. After 1 h, the solution obtained was added to a solution of amino *tert*-butyl ester **57–59** (2 mmol) in dry DMF (5 mL). The reaction was stirred at room temperature for 18 h and then concentrated under reduced pressure. The residue was dissolved with a mixture of EtOAc (10 mL) and water (5 mL), the organic phase dried over $Na_2SO_4$ and evaporated to dryness in vacuo, and the resulting crude residue purified by flash chromatography.

**General Procedure J for the Synthesis of 30–32.** A solution of the ester **60–62** (1 mmol) in $CF_3COOH$ (1 mL) was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the resulting residue was suspended in $CH_2Cl_2$ (3 mL), collected by filtration and triturated with ethyl ether (5 mL) to obtain **30–32**.

**General Procedure K for the Synthesis of Compounds 13–22.** A solution of *N*-deformyldistamycin A dihydrochloride (**33**) (263 mg, 0.5 mmol) in dry DMF (5 mL) was cooled to 5 °C and *N,N*-diisopropylethylamine (86 $\mu$L, 0.5 mmol) was added. After 10 min, the acid **23–32** (0.6 mmol, 1.2 equiv) and then EDC (192 mg, 1 mmol) were added. The reaction mixture was stirred at room temperature for 18 h, then 2 N hydro-

chloric acid was added until pH = 4. The solvent was evaporated in vacuo and the crude residue purified by flash chromatography to yield an oil, which was precipitated from MeOH/diethyl ether.

**Biological Testing in Vitro Using L1210 Cells.** The murine lymphocytic L1210 leukemia cell line was obtained from the American Type Culture Collection (ATCC). All the tested compounds were dissolved in DMSO at 1 mg/mL immediately before use and diluted in medium before addition to the cells. The murine lymphocytic leukemia cells L1210, L1210/DX and L1210/tallimustine were grown in vitro as a stationary suspension culture in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (Flow, Irvine, U.K.), 2 mM L-glutamine (GIBCO), 10 mM $\beta$-mercaptoethanol, 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. To determine survival after compound exposure, exponentially growing L1210 cells were continuously exposed to various concentrations of compounds for 48 h, after which the cytotoxic activity of the compounds was evaluated by counting surviving cells using an electronic cell counter ZBI (Coulter Counter Electronics, Hialeah, FL). The cytotoxic activity of the compounds was calculated from dose−response curves and expressed as $IC_{50}$ (concentration of test compound to reduce the cell number to 50% of that obtained with untreated control cells). All experiments were repeated at least twice. For each compound concentration, duplicate cultures were used. Vehicle or solvent controls were run with each experiment.

**Biological Tests in Vivo.** DBA/2N female mice were used for implanting with the murine L1210 leukemia. For experiments with leukemia CD2F1 female mice were used. C57B16 female mice were used for implanting with the murine reticulosarcoma M5076. Charles River Italia (Calco, Como, Italy) supplied all mice. Mice were 8−17 weeks and weighed 20−22 g at the time of tumor implantation. Animal health was monitored by serological testing; the animals were free of infectious pathogens, including mouse hepatitis virus, Sendai virus and *Mycoplasma pulmonis*, during the course of experimentation. All compound solutions were prepared immediately before use and given intravenously (iv) in a volume of 10 mL/kg of body weight. The vehicle used in preparation of solutions consisted of 10% Tween 80 and 90% saline.

L1210 murine leukemia (originally obtained from the National Cancer Institute, Frederick, MD) was maintained in vivo by continuous ip passage ($10^6$ cells/mouse), for experiments $10^5$ cells/mouse, 10 mice/group, were injected ip or iv. Compounds were administered iv or ip at day 1 after tumor cell injections. A dose−response was determined in all experiments. Toxicity was evaluated on the basis of the gross autopsy findings and the weight loss, mainly in terms of reduction of spleen and liver size.

M5076 reticulosarcoma was maintained in vivo by im serial transplantation. For experiments, $5 \times 10^5$ cells were injected im in the flank of C57B16 female mice. Animals were 8−10 weeks old at the beginning of the experiments. Compounds were administered iv at day 3, 7, and 11 after tumor implantation. Survival time of mice and tumor growth were calculated and activity was expressed in terms of %T/C and %TI. %T/C = median survival time treated group vs median survival time untreated group × 100. The survival time of control mice injected iv with L1210 is 6 days, while for ip injected mice it is 8−10 days. %TI = % inhibition of tumor growth with respect to control. Compounds were considered active if the %T/C value was ≥125.

**Target DNA, Oligonucleotide Primers, and Arrested PCR.** The sequences of ER[24,25] and Ha-ras[26] primers used for PCR[27] were the following: ER-forward, 5′-GACGCATGATATACTTCACC-3′; ER-reverse, 5′-GCAGAATCAAATATCCAGATG-3′; Ha-ras forward, 5′-AGACGTGCCTGTTG GACATC-3′; Ha-ras reverse, 5′-CGCATGTACTGGTCCCGCAT-3′. Taq DNA polymerase was purchased from FINNZYMES OY (Espoo, Finland) and added at 2.5 U/25 $\mu$L final concentration. When using FINNZYMES Taq DNA polymerase, distamycin A was found to inhibit ER amplification when present at 2−10 $\mu$M final concentrations, depending upon the type of target DNA (genomic DNA, recombinant plasmid, PCR product). For PCR-mediated amplification the target DNA was 20 ng of genomic DNA or 2 ng of pBLCAT1-ERCAT8; PCR buffer, Taq DNA polymerase and the four dNTPs were added as elsewhere described.[21] Conditions of PCRs were: denaturation, 92 °C, 1 min; annealing, 55 °C (ER) or 62 °C (Ha-ras), 1 min; elongation, 72 °C, 1 min (25 cycles). The effects of DNA-binding drugs were analyzed after incubating target DNA at room temperature, for 5 min, with increasing amounts of the compounds followed by PCR. Amplified DNA was analyzed by electrophoresis on 2% agarose gels. For sequence analysis of arrested PCR we first prepared ER target DNA by PCR. After incubation for 1 h at 37 °C with the distamycin analogues, samples were heated at 90 °C for 5 min and PCR was performed using the $^{32}$P-labeled ER reverse PCR primer (5′-GCAGAATCAAATATCCAGATG-3′). After PCR, each reaction was resuspended in 5 $\mu$L of loading dye (0.1% xylene−cyanol, 0.1% bromophenol blue, 0.1 M NaOH:formamide 1:2) and electrophoresed through a sequencing gel as described.[21]

**Direct DNA Fragmentation Assay.** A $^{32}$P-labeled ER PCR product was produced using a $^{32}$P-labeled ER reverse PCR primer (5′-GCAGAATCAAATATCCAGATG-3′). After production of the ER PCR probe, an aliquot was incubated in 50 $\mu$L of 0.1 × SSC in the presence of DNA-binding drugs. After 5-h incubation at room temperature the samples were heated at 90 °C for 30 min and ethanol was precipitated. Each reaction was resuspended in 5 $\mu$L of loading dye (0.1% xylene−cyanol, 0.1% bromophenol blue, 0.1 M NaOH:formamide 1:2) and electrophoresed through a sequencing gel as described.[21]

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**Supporting Information Available:** Experimental procedures and $^1$H NMR spectra for compounds **13−32**, **34**, and **38−62**. This material is available free of charge via the Internet at http://pubs.acs.org.

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